IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:		Tariq M. RANA
Application Number	:	10/722,176
Filing Date	:	November 24, 2003
Title	:	DELIVERY OF siRNAs
Attorney Docket No.	:	20336-00016
Examiner	:	Kimberly Chong
Group Art Unit	:	1635

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SECOND DECLARATION OF TARIQ M. RANA UNDER 37 C.F.R. §1.132

I, Tariq M. Rana, hereby make the following declaration:

1. I am the inventor of the invention described in U.S. patent application 10/722,176 filed November 24, 2003 ("the '176 application"), and U.S. provisional patent application 60/430,520, filed on November 26, 2002 ("the '520 application").

2. The '176 application as filed (and published as US 2004/0204377 A1 on October 12, 2004) describes the use of a delivery mixture comprising a delivery agent consisting of a dendrimer mixed with a nucleic acid capable of mediating RNA interference in Example 1 (paragraph [0102] of the published application and FIGS. 1A and 1B), Example 2 (paragraph [0103] of the published application and FIG. 2), and Example 7 (paragraph [0111] of the published application and FIGS. 9A –9I).

3. The corresponding description of the use of a delivery mixture comprising a delivery agent consisting of a dendrimer mixed with a nucleic acid capable of mediating RNA interference can be found in U.S. provisional patent application no. 60/430,520 in Example 2 (page 19 line 23 to page 20, line 31 and FIGS. 1A and 1B), Example 3 (page 21, lines 1-21 and FIG. 2), and Example 8 (page 24, line 29 to page 25, line 14 and FIGS. 9A –9I).

4. Experiments corresponding to the description of the use of a delivery mixture comprising a delivery agent consisting of a dendrimer mixed with a nucleic acid capable of mediating RNA interference were carried out in my laboratory by Ya-Lin Chiu under my direction and supervision.

5. Exhibits 1-11 are copies of notebook entries from the laboratory notebook of Ya-Lin Chiu, titled "Delivery Method and Localization of siRNA," for the period October 10, 2002 through February 11, 2003. Exhibits 1-11 are notebook entries of experiments carried out during the period October 21, 2002 through November 19, 2002.

a. Exhibit 1 is an outline of experimental protocol used in experiments dated 10/21/02 – 10/25/02 for determination of transfection efficiency of siRNA by various delivery agents, including PAMAM dendrimer and lipofectamine.

b. Exhibit 2 is a description of the PAMAM dendrimer used in experiments. A PAMAM G4 dendrimer in a 90 μg/μl methanol solution was used.

c. Exhibit 3 is flourescence level results of siRNA transfection experiments carried out 10/23/02.

d. Exhibit 4 is a computer printout of fluorescence results of transfection experiments using PAMAM G4 dendrimer and Lipofectamine, and carried out 10/23/02.

e. Exhibit 5 is graph results of transfection experiments introducing Cy3-labeled EGFP duplex siRNAs using PAMAM G4 dendrimer and Lipofectamine carried out 10/23/02. A note in the page indicates the product code number in the Aldrich catalog for the PAMAM Generation 4 dendrimer used in experiments. Exhibit 8 is a graph summary of results of similar transfection experiments introducing Cy3-labeled EGFP duplex siRNAs carried out 10/23/02.

f. Exhibit 6 is protein levels of lysates from cells transfected with CDK9 siRNA by PAMAM dendrimer 10/29/02.

g. Exhibit 7 is immunoblot results of lysates from cells transfected with CDK9 siRNA by PAMAM dendrimer. Exhibit 9 is also immunoblot results of lysates from cells transfected with CDK9 siRNA by PAMAM dendrimer on 10/25/02.

h. Exhibit 10 and Exhibit 11 depict results of microscopic examination of HeLa cells transfected with Cy3-SS/AS siRNA by Lipofectamine or PAMAM, respectively on 11/19/02.

6. The notebook pages of Exhibit 2 and Exhibit 5 evidence use of generation 4 PAMAM dendrimer for transfection experiments. Each of the experiments carried out during the period 10/21/02 through 11/19/02 using a PAMAM dendrimer as a delivery agent used generation 4 PAMAM dendrimer.

7. Exhibits 1-11 are experiments and results corresponding to Example 1 (paragraph [0102] of the published application and FIGS. 1A and 1B), Example 2 (paragraph [0103] of the published application and FIG. 2), and Example 7 (paragraph [0111] of the published application and FIGS. 9A –9I) of the '176 application.

a. Example 1 and FIG. 1A and FIG. 1B correspond to the experiments and results depicted in Exhibits 1, 3, 4, 5, and 8.

b. Example 2 and FIG. 2 correspond to the experiments and results in Exhibits 6, 7, and 9.

c. Example 7 and FIG. 9 correspond to the experiments and results in Exhibit 11.

8. Exhibits 1-11 are experiments and results corresponding to Example 2 (page 19 line 23 to page 20, line 31 and FIGS. 1A and 1B), Example 3 (page 21, lines 1-21 and FIG. 2), and Example 8 (page 24, line 29 to page 25, line 14 and FIGS. 9A –9I) of U.S. provisional patent application no. 60/430,520.

a. Example 2 and FIG. 1A and FIG. 1B correspond to the experiments and results depicted in Exhibits 1, 3, 4, 5, and 8.

b. Example 3 and FIG. 2 correspond to the experiments and results in Exhibits 6, 7, and 9.

c. Example 8 and FIG. 9 correspond to the experiments and results in Exhibit 11.

9. The experiments described in the '176 application (Example 1 (paragraph [0102] of the published application and FIGS. 1A and 1B), Example 2 (paragraph [0103] of the published application and FIG. 2), and Example 7 (paragraph [0111] of the published application and FIGS. 9A –9I) of the '176 application) used a generation 4 PAMAM dendrimer as a delivery agent mixed with a nucleic acid capable of mediating RNA interference.

10. The experiments described in U.S. Provisional application 60/430,520 (Example 2 (page 19 line 23 to page 20, line 31 and FIGS. 1A and 1B), Example 3 (page 21, lines 1-21 and FIG. 2), and Example 8 (page 24, line 29 to page 25, line 14 and FIGS. 9A –9I) of U.S. provisional patent application no. 60/430,520) used a generation 4 PAMAM dendrimer as a delivery agent mixed with a nucleic acid capable of mediating RNA interference.

11. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Respectfully Submitted,

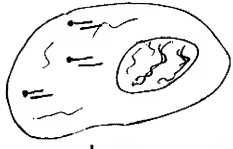
Date: Oct 26, 2007

Tariq M. Rana

10/21/02 ~ 10/23/02 Determine transfection efficiency
Using cy3-labeled siRNA as indicator

EGFP 5'cy3-SS/AS Duplex

↓ Transfection by
1. Lipofectamine
2. Nanoparticle # 1
3. PAMAM

Hela cell  on 60mm plates

↓ 6hr incubation at 37°C

Washed 3x by 5mL PBS

↓

Qiagen DNA/RNA Extration kit

pellet
protein
Removed by strong denaturing reagent
followed by ϕ 13000rpm 20 min
4°C

Solution
containing DNA
RNA
Small RNA

↓

Isopropanol
precipitation
4°C. 13000rpm 30 min

↓

Redisolve pellet in H2O
(heat at 70°C 10 min)

↓

Detect cy3 signal
by Fluorometer

Ya-Lin Chen

EXHIBIT 1

PMAM-G4

10/21/02

10g/110 mL.

90 ug/uL. in Methanol.

EXHIBIT 2

10/23/02

102302-1

	GFP
1	18560
2	10690
3	18220
4	19400
5	11650
6	10570
7	8500
8	8867
9	7982
	11080
11	10780
12	4126
13	5962
14	4698

102302-2

	EGFP	RFP2
*		
2	272300	104300
3	53180	101900
4	252200	98710
5	266600	104300
6	286400	119000
7	364800	160300
8	587700	276300
9	594500	291400
102302-3		
10	174700	87680
11	132900	84660
12	89570	86980
13	58490	91380
14	54000	91050
15	35040	92920
16	26180 ~~25990~~	106900
17	25970 ~~28160~~	115800
18	28160	135900
19	22080	120500

102302-4

	EGFP	RFP
20	262800	97460
21	261400	98850
22	218700	84620
23	200700	84480
24	208600	90110
25	229700	113900
26	188300	108000
27	203800	124400
28	140300	91130
102302-5		
29	249200	98710
30	244900	97150
31	246500	104900
32	219900	101800
33	222800	105500
34	193400	94150
35	166800	90910
36	153500	91580
37	128200	72100

102302-6

	EGFP	RFP
38	279200	111900
39	275500	103900
40	306600	110500
41	240200	83860
42	264900	104100
43	294500	120000
44	230700	95480
45	234900	94940
46	252700	93130
47	211100	81610
48	123300	45220
49	52850x2	18260x2
~~50~~	256900	90710
51		
52	203400	66640
53	185300	47270
54	48440x2	14249x2

See Detail in Modified SiRNA Effect

Ya-Lin Chiu

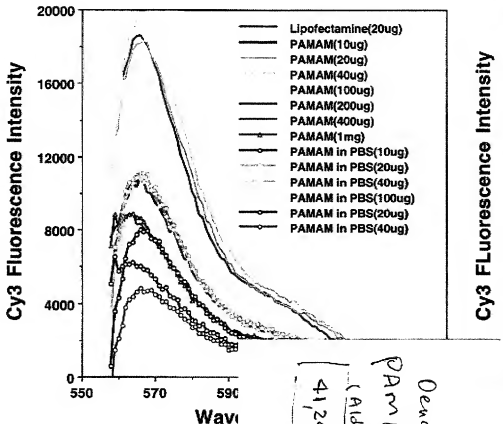
EXHIBIT 3

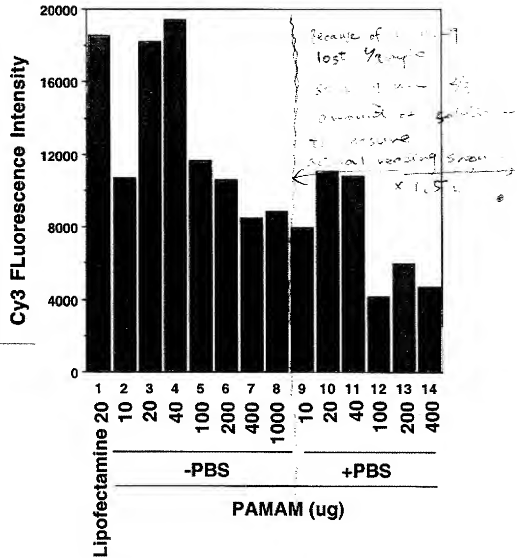
	Cy3 FLuorescence Intensity	Treatment	Relative siRNA uptake Efficienc
1	18560.000	Lipofectamin (20ug)	1.000
2	10690.000	PAMAM(10ug)	0.576
3	18220.000	PAMAM(20ug)	0.982
4	19400.000	PAMAM(40ug)	1.045
5	11650.000	PAMAM(100ug)	0.628
6	10570.000	PAMAM(200ug)	0.570
7	8500.000	PAMAM(400ug)	0.458
8	8867.000	PAMAM(1mg)	0.478
9	7982.000	PAMAM 1 in PBS (10ug)	0.430
10	11080.000	PAMAM 1 in PBS (20ug)	0.597
11	10780.000	PAMAM 1 in PBS (40ug)	0.581
12	4126.000	PAMAM 1 in PBS (100ug)	0.222
13	5962.000	PAMAM 1 in PBS (200ug)	0.321
14	4698.000	PAMAM 1 in PBS (400ug)	0.253

EXHIBIT 4

102302

Comparison Analysis of PAMAM to Lipofectamine
(EGFP 5'Cy3-SS/AS siRNA as transfection efficiency indicator)





Dendrimer PAMAM Generation 4
(Alderich)
41,244-9

EXHIBIT 5 EMD

	OD750	Protein(ug/ul)	60ug	ul/60ug	Buffer
1	0.716	6.616	60.000	9.069	11.931
2	0.613	5.642	60.000	10.634	10.366
3	0.779	7.211	60.000	8.320	12.680
4	0.783	7.249	60.000	8.277	12.723
5	0.648	5.973	60.000	10.045	10.955
6	0.587	5.396	60.000	11.119	9.881
7	0.553	5.075	60.000	11.823	9.177
~~8~~	~~0.471~~	~~4.309~~	~~60.000~~	~~13.955~~	~~7.045~~
~~9~~	~~0.968~~	~~3.326~~	~~60.000~~	~~18.040~~	~~2.960~~

Mock (40ug PAMAM only)
100nM cdk9siRNA by 20ug
40ug
100ug PAMAM
200ug
400ug
1mg

EXHIBIT 6

10/25/02 ~ 10/30/02

→ From 10/21/02 and 10/25/02 experiment.
we known that PAMAM Dentrimer can sent siRNA into the cell.
but cannot sent the reporter plasmid in (size constraint?)
so ⇒ cannot use dual fluorescence assay to get Qutitation data

→ Silencing of cdk9 expression by PAMAM mediated transfection

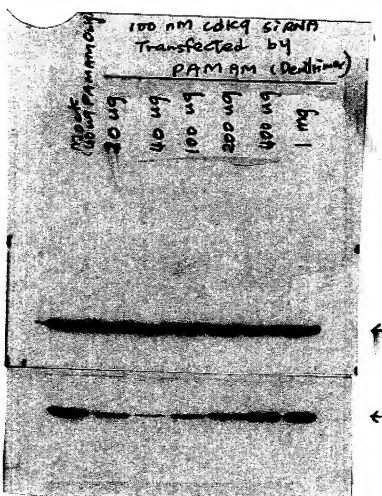
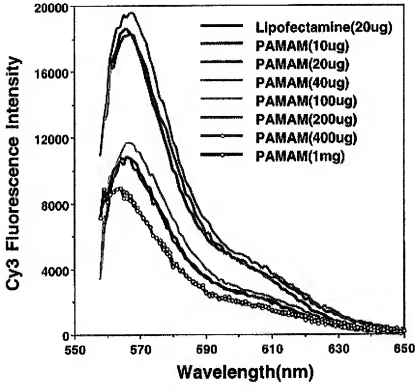


EXHIBIT 7

Summary of PATENT 10/23/2002

(A)

Fig 1



10/23/2002

(B)

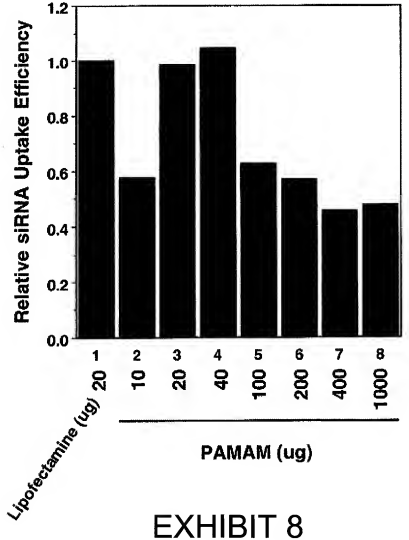


EXHIBIT 8

Yan Lin Chen

Summary of PAMAM

10/25/2002

Fig 2

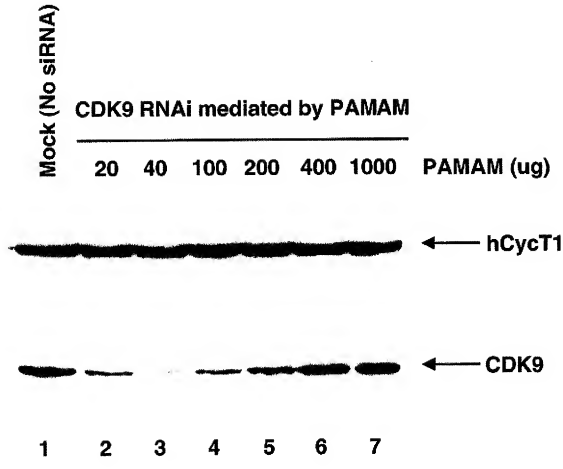


EXHIBIT 9

TM

Ya-Lin Chen

11/19/02

Uptake of siRNA by HeLa Cells (20ug/1mL Lipofectamine-Mediated Transfection, 6h)(111902)

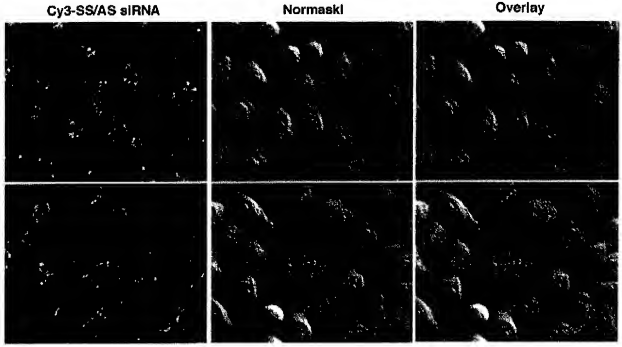


EXHIBIT 10

11/19/2002
Ya-Lin Chiu

11/19/02

Uptake of siRNA by HeLa Cells (40ug/1mL PAMAM-Mediated Transfection, 6h)(111902)

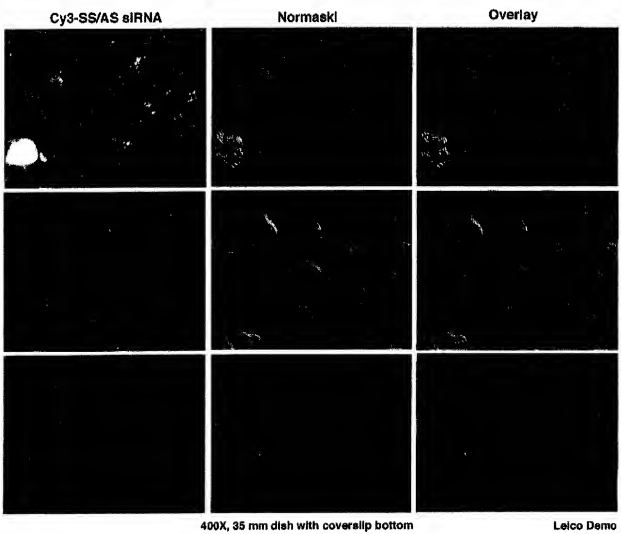


EXHIBIT 11

11/19/2002